Use of gangliosides and other substances for modulating sphingolipid-cholesterol microdomains

Description

The invention relates to the use of gangliosides and other substances for preparing an agent for modulating sphingolipid-cholesterol microdomains.

Sphingolipid-cholesterol microdomains, which are also termed sphingolipid-cholesterol rafts, are lateral arrangements of specific lipids, in particular sphingolipids including gangliosides and cholesterol, and also proteins, in cell membranes (K. Simons et al., Nature, Vol. 387 (1997), 569-572; T. Friedrichson et al., Nature, Vol. 394 (1998), 802-805). These microdomains are small and highly dynamic structures which are formed by attractive forces between sphingolipids and cholesterol. A variety of proteins are associated with the sphingolipid-cholesterol structures, including GPI-APs (glycosylphosphatidyl anchor proteins), kinases of the Src family, influenza virus hemagglutinin (HA) and caveolin-1. Most of the proteins which are associated with the sphingolipid-cholesterol rafts contain post-translational lipid modifications, with GPI-anchored proteins being anchored in the cell membrane by way of a lipid unit whereas kinases of the Src family, NO synthases (NOS), HA and caveolin are acylated. The proteins which are associated with the rafts are normally only poorly soluble in cold, nonionic detergents, such as Triton X-100, a property which is possibly to be attributed to their lipophilic character. Complexes which are insoluble in Triton have been defined, with regard to their function, as being rafts, which are specifically enriched at specific lipids, in particular sphingolipids, including gangliosides, and cholesterol, whereas other lipids, such as glycerophospholipids, are

selectively lacking in the Triton extracts (Brown et al., Trends Cell Biol. 2 (1992), 338-343; Fiedler et al., Biochemistry 32 (1993), 6365-6373).

The existence of sphingolipid-cholesterol microdomains or rafts in living cells, in particular the existence of microdomains of GPI-anchored proteins in living cells, which domains can be influenced by administering cholesterol, has recently been demonstrated by means of a chemical crosslinking approach (Friedrichson et al., see above) and a fluorescence energy transfer method (Varma et al., Nature 394 (1998), 798-802). It has furthermore been postulated that, as platforms to which proteins can bind, the rafts play an important role in membrane transport and signal transmission properties.

An object of the invention was therefore to provide an agent for modulating sphingolipid-cholesterol microdomains (and in particular their constitution and/or properties) in order to be able to selectively exert an effect on the processes which are influenced by these microdomains.

According to the invention, this object is achieved by using gangliosides and other substances for preparing an agent for modulating sphingolipid-cholesterol microdomains.

Within the context of the present invention, it has been possible to establish that proteins which are associated with sphingolipid-cholesterol microdomains, in particular GPI-anchored proteins, are present in the microdomains in the form of crosslinked clusters on the surface of living cells. Adding gangliosides and other substances, such as cholesterol sulfate, inhibits the crosslinking of proteins which are associated with the rafts, in particular GPI-anchored proteins, and, in

addition to this, increases their solubility in detergents.

The administration of gangliosides and other substances consequently leads to a change in the structural constitution of the microdomains, such that these gangliosides and other substances can be used for modulating sphingolipid-cholesterol microdomains and the processes which take place in these domains.

It is assumed that the administration of gangliosides and other substances displaces proteins, in particular GPI-anchored proteins, from the sphingolipid-cholesterol microdomains as a result of the attractive forces between the anchor group and the surrounding lipids being disrupted. The consequence of this is that, when gangliosides and other substances are added, the proteins which are present in the sphingolipid-cholesterol microdomains are leached out of their lipid environment and are no longer associated with the rafts.

As a consequence of this, the addition of exogenous gangliosides alters, for example, the solubility of GH-DAF (growth hormone (GH) which is linked to the GPI anchor of the DAF (decay accelerating factor)), a form of growth hormone which is glycosylphosphatidyl inositol-anchored, in detergents. It is known that GPI-anchored proteins are essentially insoluble in nonionic detergents (Brown et al., Cell 68 (1992), 533-544) whereas a decrease in the cholesterol content in cells increases the detergent solubility of GPI-APs (Cerneus et al., J. Biol. Chem. 268 (1993), 3150-3155; Hanada et al., J. Biol. Chem. 270 (1995), 6254-6260). This means that the forces of interaction between the lipid constituents and the proteins in the microdomains are greater than the solubilizing forces due to nonionic detergents. However, if the microdomains are altered or

destroyed by removing the constituent cholesterol, the forces of interaction between the lipids and the proteins are then diminished and the proteins can be leached out of the rafts by nonionic detergents.

Surprisingly, it has now been found that the opposite applies when gangliosides and other substances, which are preferably also a lipid constituent of the rafts, are added, i.e. that the addition of gangliosides to cells increases the detergent solubility of GH-DAF. This observation is unexpected since it would be assumed that an increase in the concentration of a lipid which is present in the sphingolipid-cholesterol microdomains would stabilize these domains. In fact, however, the addition of gangliosides disrupts the crosslinking of the proteins and increases the detergent solubility of GH-DAF. Gangliosides consequently bring about a modulation of the microdomains, in particular a displacement of proteins, in particular GPI-APs, from the rafts.

This is confirmed by the fact that gangliosides disrupt the copatching of HA and GH-DAF. The copatching of two microdomain components is a consequence of the coalescence of a common lipid microdomain.

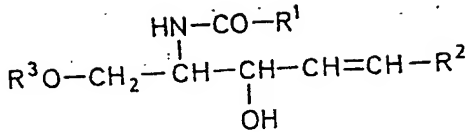
The gangliosides preferably modulate the microdomains specifically and reversibly, i.e. when the gangliosides and other substances are removed, the original, unaltered structure of the microdomains is obtained once again.

According to the invention, therefore, preference is given to using gangliosides to influence the location of proteins on sphingolipid-cholesterol microdomains, in particular the location of anchor proteins, acylated proteins, Src kinases and/or cholesterol-anchored or GPI-anchored proteins. Adding gangliosides alters the

arrangement of proteins which are present in sphingolipid-cholesterol microdomains, with this leading to a modulation and/or change in structure and/or constitution of the sphingolipid-cholesterol microdomains. Particular preference is given to the protein clusters being disassembled.

The gangliosides which are employed in accordance with the invention are preferably selected from bovine brain gangliosides, GM_1, GD1a, GD1b, GD3, GM2, GM3, GQ1a, GQ1b and and/or globosides. Particular preference is given to using exogenous gangliosides and their derivatives, such as unsaturated sphingosine or ceramide containing short or unsaturated fatty acids which are already constituents of the sphingolipid-cholesterol microdomains to be treated. However, cholesterol sulfate and other cholesterol derivatives can also be employed.

The terms ganglioside and/or ganglioside derivatives, which are used herein, encompass, in particular, glycolipids which occur as structural elements of cell membranes in nerve tissue. The gangliosides and/or ganglioside derivatives preferably contain several monosaccharide units per molecule. Examples of suitable monosaccharide units which can be contained in the gangliosides and/or ganglioside derivatives are D-galactose, N-acetyl-D-galactosamine, D-glucose and N-acetylneuraminic acid. Particular preference is given to gangliosides which are derivatives of sphingosine (2-amino-4-octadecene-1,3-diol, sphing-4-enine), with, in particular, sugar residues being bonded on by way of the oxygen on the C-1 and a short (in particular C_2-C_{18}) fatty acid, which can be saturated or unsaturated, being bonded by way of the nitrogen on the C-2. Preference is furthermore given to the gangliosides which are a derivative of a ceramide. Ceramides are lipophilic amides of the formula



where R^1 is a long-chain fatty acid residue, in particular a C_6-C_{30}, more preferably a C_8-C_{24}, fatty acid residue, R^2 is a long-chain alkyl residue, in particular a C_6-C_{30}, more preferably a C_8-C_{24}, alkyl residue, and R^3 is H. In the ceramide-containing gangliosides, R^3 is preferably replaced with sugar residues (glycosides).

Consequently, ganglioside derivatives preferably comprise ceramide and its derivatives and related compounds including sphingosines and gangliosides and their derivatives, with it also being possible for one of the following functional groups to be substituted or added on the backbone chain, in particular the ceramide backbone chain: a halide atom, bonded to an alkyl, alkenyl, alkynyl or aryl radical, an alcohol group (primary, secondary or tertiary), an ether group, a carbonyl function (e.g. aldehyde or ketone), a carboxylic acid group, a carboxylic anhydride group, a carbamoyl group, a haloformyl group, a cyano group, an ester group, including a lactone group, a benzyl, phenyl, tolyl, tosyl or sulfonyl group, an amino group (primary, secondary or tertiary), an isocyanate, a cyanate, a thioisocyanate, a thiocyanate, a carbamate, an azide, a diazo group or a quinone group. Other groups which can be bonded to the cholesterol unit include an oligopeptide comprising from 2 to 20, preferably from 8 to 12, in particular 8, 10 or 12 amino acid residues, an oligonucleotide, or individual amino acids, monosaccharides, disaccharides or polysaccharides.

All the substances which have been formed from cholesterol by substituting or adding groups come within the term cholesterol derivatives. In particular, cholesterol derivatives are understood as being compounds which are formed from cholesterol in only one reaction step and are chemically closely related to cholesterol. Cholesterol derivatives include cholesterol sulfate, cholesterol thiosulfate and all cholesterol derivatives in which organic groups are substituted or added onto the cholesterol unit. Suitable substituted or added groups comprise: a halide atom, bonded to an alkyl, alkenyl, alkynyl or aryl radical, an alcohol group (primary, secondary or tertiary), an ether group, a carbonyl function (e.g. aldehyde or ketone), a carboxylic acid group, a carboxylic anhydride group, a carbamoyl group, a haloformyl group, a cyano group, an ester group, including a lactone group, a benzyl, phenyl, tolyl or tosyl, a sulfonyl group, an amino group (primary, secondary or tertiary), an isocyanate, a cyanate, a thioisocyanate, a thiocyanate, a carbamate, an azide, a diazo group or a quinone group. Other groups which can be bonded to the cholesterol unit include an oligopeptide comprising from 2 to 20, preferably from 8 to 12, in particular 8, 10 or 12, amino acid residues, an oligonucleotide, individual amino acids, monosaccharides, disaccharides or polysaccharides.

Examples of preferred cholesterol derivatives are cholesterol sulfate and cholesterol molecules which are derivatized on the OH function.

The present invention furthermore provides a process which can be used for monitoring and observing the direction of targeting and processing of the abovementioned active compounds, that is the gangliosides, gangloside derivatives and cholesterol derivatives, by attaching a detectable group, for

example a fluorescent group, to one of the previously enumerated compounds, for example by means of substitution or addition.

According to the invention, the term "modulation" covers any structural and chemical change in the microdomains, in particular a change in the structural constitution and/or in the combination of the individual constituents of the microdomains. For its part, the modulation of the sphingolipid-cholesterol microdomains preferably brings about a change in the membrane transport, signal transmission and/or cell adhesion properties and/or a change in enzymic processes. These effects are caused by using gangliosides and other substances for the modulation, that is, in particular, for altering the structure of sphingolipid-cholesterol microdomains. Thus, signal transmission properties can be influenced by using exogenously added gangliosides and other substances to alter the sphingolipid-cholesterol rafts. In addition to this, it has been observed that the addition of gangliosides to cells alters the degree of autophosphorylation of many kinases. The sphingolipid-cholesterol microdomains consequently constitute platforms for a variety of processes, in particular for signal transmission processes, which, according to the invention, can be influenced by using gangliosides to modulate the microdomains. Modulation of the sphingolipid-cholesterol microdomains can also prevent the phagocytosis of bacteria and parasites in mammalian cells. Modulation of the sphingolipid-cholesterol microdomains can also prevent the uptake of viruses in mammalian cells as well as their transport and release.

GPI-anchored proteins associate, for example, with tyrosine kinases of the Src family (Brown, Trends Cell Biol. 2 (1992), 338-342). In addition, cell signal proteins are concentrated in the caveolae, which are

plasma membrane invaginations which are involved in the sequestering and organizing of raft lipid domains. Many signal transmission molecules, such as PDGF (platelet derived growth factor), EGF (epidermal growth factor), protein kinase C and the insulin receptor, are associated with sphingolipid-cholesterol rafts or caveolae. The signal transmission pathways which are mediated by these molecules can be inhibited by the exogenous addition of gangliosides and other substances. It has now been observed that gangliosides modify these signal transmission pathways by, for example, effecting a cluster disassembly of protein-containing microdomains, in particular of GPI-AP-containing microdomains.

Still other signal transmission processes are dependent on the arrangement of lipid and protein components in the rafts. A modulation, in particular a disassembly of the rafts by the exogenous administration of gangliosides can be brought about, for example, in order to antagonize a microdomain-dependent signal transmission. These signal transmission processes include, inter alia, an antibody-induced patching of GPI-anchored PLAP (placental alkaline phosphatase), which leads to an accumulation of the Src-like tyrosine kinase fyn (Harder et al., J. Cell. Biol. 141 (1998), 929-942). Furthermore, physiological reactions which are induced by microdomain clusters have been described in the case of lymphocytes (Brown, Curr. Opin. Immunol. 5 (1993), 349-354). Thus, an antibody-induced crosslinking of GPI-anchored proteins leads to the activation of T lymphocytes (Thomas et al., J. Biol. Chem. 267 (1992), 12317-12322), and the crosslinking of the IgE receptor FcεRI causes an allergic reaction including secretion of histamine in mast cells (Holowka et al., Ann. Rev. Biophys. Biomol. Struct. 25 (1996), 73-80; Field et al., J. Biol. Chem. 272 (1997), 4276-4280). Furthermore, gangliosides can be used for

treating diseases which are connected to a cell movement, such as the control of tumors and/or metastases. Tumor cells use the abovementioned mechanism in order to evade the immune response. Raft-mediated processes, such as the activation of T cells in the presence of tumor cells, can consequently be influenced by gangliosides. The use, according to the invention, of gangliosides and other substances makes it possible to modulate, and thereby selectively influence, functions which are affected by sphingolipid-cholesterol microdomains.

It has furthermore been ascertained that an increase in the autophosphorylation of various protein kinases can be observed when gangliosides are added to cells.

In addition to this, the gangliosides and other substances can be used as agents for clarifying the pathogenesis of diseases which are associated with activities at sphingolipid-cholesterol microdomains. Thus, it has been recently demonstrated that a decrease in the cholesterol of hippocampus neurons inhibits the secretion of β-amyloid peptide, from which it follows that the sphingolipid-cholesterol microdomains play an important role in the proteolytic processing of the amyloid precursor protein (APP) in Alzheimer's disease (Simons et al., Proc. Natl. Acad. Sci. USA 95 (1998), 6460-6464). The transformation of the GPI-anchored prion protein into the scrapie protein isoform has likewise been suggested to be connected with sphingolipid-cholesterol microdomains (Taraboulos et al., J. Cell Biol. 129 (1995), 121-132). Preference is therefore given to using the gangliosides such that the modulation of the sphingolipid-cholesterol microdomains brings about a change in the proteolysis of the amyloid precursor protein in Alzheimer's disease or a modification of a prion protein.

The modulation, according to the invention, of sphingolipid-cholesterol microdomains can be of interest for both and therapeutic, preventive and diagnostic purposes. In this connection, the gangliosides are preferably administered in a dose of 3, preferably of at least 5 up to 30, and preferably up to 20 mg per kg per day.

In an in vitro system (cell culture), the dose which is used experimentally to elicit a reaction is 1 mg/ml for from about 10 to 12 hours. Taking into account the buffering effect of blood, plasma and cells in animals or humans, this indicates a dose range of from 3 to 30 mg/kg per day.

The invention is explained further by the following examples and the enclosed figures.

Figure 1

shows that gangliosides inhibit the crosslinking of GPI-anchored proteins, in particular of GH-DAF, in living cells. MDCK GH-DAF cells (MDCK: dog, cocker spaniel, kidney) were incubated, at 37°C for 1 hour, with 0, 10, 50 and 100 µM GM_1 (A) or with 10, 50 and 100 µM bbG (B). CHO FR-GPI cells were incubated, at 37°C for 1 hour, with 100 µM bbG or with 100 µM GM_1. The cells were subsequently crosslinked with BS^3. The proteins were separated using 5-15% SDS-PAGE and, after Western blotting, detected using an anti-GH antibody, followed by ECL (electrochemoluminescence). The autoradiographs were scanned, and the intensity of the immunoreactive signal is depicted as relative optical density (ROD) for 0 µM GM_1 (A, upper curve), 0 µM bbG (B, upper curve), 100 µM GM_1 (A, lower curve) and 100 µM bbG (B, lower curve).

Figure 2

shows that gangliosides inhibit the crosslinking of FR-GPI in CHO cells. CHO FR-GPI cells were incubated, at 37°C for 1 hour, with 100 µM bbG or with 100 µM GM_1. The cells were subsequently crosslinked with BS^3. The proteins were separated using 5-15% SDS-PAGE and detected, following Western blotting, using an anti-folate receptor antibody, followed by ECL (electrochemoluminescence).

Figure 3

shows the incorporation of GM_1 into the plasma membrane of MDCK GH-DAF cells. In (A), MDCK GH-DAF cells were incubated, at 37°C for 1 hour, with 1 µCi/ml tritium-containing GM_1 at a final ganglioside concentration of 100 µM and subsequently washed with BSA (bovine serum albumin) for 0, 10, 20, 30 or 45 minutes (hatched bars). The additive effect of a treatment with 0.1% trypsin for 5 minutes at 37°C, after the washing with BSA, is shown as solid bars. In (B), the crosslinking was carried out after incubating GM_1-loaded cells with BSA for 0, 10, 20, 30 or 45 minutes. SDS-PAGE, Western blotting and detection were carried out as described in figure 1.

Figure 4

shows the increase in the detergent solubility of GH-DAF due to gangliosides. In (A), MDCK GH-DAF cells were loaded with 100 µM bbG for 1 hour at 37°C, or with 100 µM NBD-C_6-HPC for 1 hour at 8°C, with 100 µM GM_1 for 1 hour at 37°C or with 100 µM bbG for 1 hour followed by a 6-hour incubation with DMEM-containing serum (A) or a treatment with 10 mM methyl-β-cyclodextrin for 1 hour at 37°C or an incubation with anti-GH for 1 hour at 12°C, followed by an incubation with a secondary antibody (B). The cells were extracted with Triton X-114 at 4°C for 30 minutes, and centrifuged, and the

GH-DAF in the soluble (S) and insoluble (I) fractions was detected as described below.

Figure 5 shows that the effect of octyl glucoside on sphingolipid-cholesterol microdomains is different from the effect of gangliosides. MDCK GH-DAF cells were incubated for 1 hour with 5 and 10 mM octyl glucoside and crosslinked with BS^3. A slight decrease in crosslinking was only observed when using 10 mM octyl glucoside. The membranes remained intact at all the concentrations investigated.

Figure 6 shows that the inhibition of the crosslinking of GH-DAF is a specific property of gangliosides. In (A), MDCK GH-DAF cells were incubated, at 8°C for 30 minutes, with 0, 10, 50 or 100 µM NBD-C_6-HPC before they were crosslinked with BS^3. SBS-PAGE, Western blotting and detection were carried out as described in figure 1. In (B), MDCK GH-DAF cells were incubated with 0 or 100 µM bbG for 1 hour, followed by a 6-hour incubation with a medium containing serum (DMEM/FCS). The cells were crosslinked, followed by SDS-PAGE, Western blotting and detection, as explained in figure 1.

Figure 7 shows that treatment with a ganglioside does not alter the immunofluorescence pattern of GH-DAF. MDCK GH-DAF cells were incubated, at 37°C for 1 hour, without any addition (A, D), with 100 µM bbG (B) or with 10 mM CD (C). The cells were fixed and labeled with an anti-GH antibody, followed by a Cy3 conjugated anti-sheep IgG (A, B and C), or crosslinked, prior to being fixed, in an antibody-induced manner.

Figure 8

shows that the loading of MDCK GH-DAF cells with gangliosides alters the autophosphorylation status. MDCK GH-DAF cells were loaded with bbG or GM_1 at 37°C for 1 hour or crosslinked with 0.5 mM BS^3 at 4°C for 45 minutes. After lyzing, aliquots of the samples were separated on a 5-15% gel and the in-gel assay was carried out for the protein kinase activity. Bands whose intensity increased in association with the ganglioside treatment are marked with an arrow. Bands which only became visible after treatment with a ganglioside are marked with an arrow head.

Figure 9

shows the inhibition, by gangliosides, of the copatching of influenza HA and GH-DAF. MDCK GH-DAF cells were infected with the influenza HA virus and then incubated, at 37°C for 1 hour, with DMEM (A-F) or 100 µM GM_1 in DMEM (G-L). Following a subsequent treatment with a mixture of monoclonal anti-HA mouse antibodies and polyclonal anti-GH sheep antibodies, at 4°C, the patching was detected using labeled secondary Cy-3-anti-sheep (red) and FITC-anti-mouse (green) antibodies. The panels in the left-hand column show the distribution of GH while those in the middle column show the distribution of HA and those in the right-hand column show the combination of both signals. Panels D-F in each case show a detail from panels A-C while panels J-L show a detail from panels G-I. The lines in F and L correspond to 2 µM, while those in C and I correspond to 8 µM.

Examples

Example 1

Inhibiting the crosslinking of GPI-anchored proteins by exogenously administering gangliosides

In order to analyze the organization of GPI-anchored proteins on the surface, use was made of an MDCK cell line which permanently expresses GH fused to the GPI anchor of the decay accelerating factor (GH-DAF) (Friedrichson et al., Nature 394 (1998), 802-805). The MDCK GH-DAF cells were stored at 37°C, under 5% CO_2, in DMEM which was supplemented with 10% FCS and antibiotics. The experiments were carried out on confluent or subconfluent cells which were cultured in plastic dishes. When MDCK GH-DAF cells were crosslinked chemically with bis(sulfosuccinimidyl)suberate (BS^3), Western blotting detected a distinct band at 46 kDa (dimer) and a smeared band, which extended from 60 kDa up to 300 kDa (figure 1A).

In order to investigate the influence of gangliosides on sphingolipid-cholesterol rafts, MDCK GH-DAF cells were loaded, at 37°C for 1 hour and in a serum-free medium, with various concentrations of GM_1 and crosslinked with BS^3. As can be seen in figure 1A, treating MDCK GH-DAF cells with GM_1 decreased the quantity of crosslinked GH-DAF significantly. Quantifying the immunoreactive bands showed that 73% ± 6% of the GH-DAF formed oligomers in untreated cells, while 65% ± 7%, 57% ± 7% and 49% ± 7% of GH-DAF was found to be in crosslinked species in cells which had been treated with 10, 50 and 100 µM GM_1, respectively. Incubating the cells with bovine brain gangliosides (bbG) also efficiently inhibited the formation of GH-DAF oligomers (figure 1B). When 100 µM bbG was used, the crosslinking efficiency was 51% ± 5%, as compared with 73% ± 6% in untreated cells.

In order to determine whether the ability of gangliosides to decompose GPI-AP-containing rafts is independent of the cell type, CHO cells which were permanently expressing the GPI-anchored folate receptor (FR-GPI) were treated in the same way. The CHO FR-GPI cells were cultured in a folate-free Hams F-12 medium which contained 5% FCS, hygromycin (100 µg/ml), penicillin (100 U/ml) and streptomycin (100 µg/ml). The crosslinking of CHO FR-GPI cells with BS^3 leads to the appearance of crosslinked FR-GPI products, with a crosslinking efficiency of 28% ± 3% being observed. When CHO FR-GPI cells were loaded with 100 µM GM_1 or 100 µM bbG prior to the crosslinking, the crosslinking efficiency was decreased to 2.7% ± 2.5% and 3.3% ± 2%, respectively (see figure 2).

In order to quantify the quantity of GM_1 which is incorporated into the plasma membrane, MDCK GH-DAF cells were incubated, for 1 hour, with 1 µCi/ml tritium-containing GM_1 at a final ganglioside concentration of 100 µM. For this, GM_1 was extracted, purified in accordance with known methods (Tettamanti et al., Biochim. Biophys. Acta 296 (1973), 160-170), and radiolabeled at the C-3 of the long-chain base group (Sonnino et al., J. Lipid Res. 25 (1984), 620-629). The homogeneity of the radioactive compound ^3H-GM_1 was greater than 99% and its specific radioactivity was 1.31 Ci/mmol. The cells were incubated for 1 hour in DMEM which contained 1 µCi/ml tritium-containing GM_1 at a final ganglioside concentration of 100 µM, after which the cells were washed with PBS/BSA and subsequently incubated at 37°C, for from 0 to 45 minutes, with PBS/BSA. The cells were then treated for 5 minutes, at 37°C, with 1 ml of PBS which contained 0.1% trypsin. The cell lysates were analyzed for their content of radioactive ganglioside using a liquid scintillation counter (Beckmann

Instruments, CA). The protein was determined using a protein assay reagent (BCA; Pierce, UK).

Only about 0.7% of the exogenously added ganglioside associated with the cells. It was possible to remove approximately 35% of the total associated GM_1 by incubating the cells with BSA solution (figure 3A). By subsequently treating the cells with trypsin, it was possible to detect two pools of associated GM_1. About 30% of the remaining GM_1 was released by treating with trypsin (trypsin-labile pool) while about 70% was stable (trypsin-stable pool) (figure 3A). It is assumed that the trypsin-stable pool of GM_1 corresponds to the molecules which are inserted into the plasma membrane, whereas the trypsin-labile pool consists of gangliosides which interact with proteins which protrude from the plasma membrane (cf. Masserini et al., Biochemistry 29 (1990), 697-701; Saqr et al., J. Neurochem. 61 (1993), 495-411). The trypsin-stable pool of GM_1 contained, in an enriched state, that fraction which was resistant to an extraction with Triton X-100 at 4°C. After incubating cells with 100 µM tritium-containing GM_1, 3.74 nmol of GM_1 (per mg of protein) were found in the detergent-insoluble fraction while 1.26 nmol of GM_1 were isolated from the soluble fraction, an observation which demonstrates that exogenous GM_1 accumulates in the rafts.

In order to exclude the possibility that the inhibition of the crosslinking of GH-DAF takes place as the result of the effect of gangliosides which are loosely associated with the surface, the crosslinking was carried out on GM_1-loaded cells before and after incubating with BSA. Under these two conditions, the crosslinking efficiency remained the same (see figure 3B; the variation in efficiency was less than 5% under all conditions). Consequently, gangliosides which are firmly associated with the membrane are responsible

for the inhibition of the crosslinking. In accordance with the criterion used by other authors (Kanda et al., J. Biochem. (Tokyo) 91 (1982), 1707-1718; Schwarzmann et al., Biochemistry 22 (1983), 5041-5048) it was established that 70% of the firmly associated GM_1 was incorporated into the double layer. Consequently, the contribution made by the trypsin-labile pool to the crosslinking, if it occurs at all, is very small in particular since the solubility of GH-DAF in TX-114 was substantially increased by gangliosides. This behavior cannot be explained by ganglioside micelles which bind to protruding proteins.

Example 1A

Using cholesterol sulfate to disrupt cholesterol-enriched microdomains in MDCK cells

Cholesterol sulfate-methyl-β-cyclodextrin (MβCD) inclusion complexes were prepared essentially as described by Klein et al. (1995), Biochemistry 34, p. 13784. 5 ml of an aqueous solution of MβCD (10 mg/ml) were stirred constantly, at 60°C, on a water bath and 0.15 ml of a cholesterol sulfate (CS) solution (10 mg/ml in isopropanol) was added in small portions. The solution was ultrasonicated at 40°C for 10 minutes, centrifuged at 100 000 g for 10 minutes and lyophilized. The dried inclusion complex was finally dissolved in 300 µl of water in order to yield a stock solution which was 10 mM with respect to cholesterol sulfate. MDCK GH-DAF cells (10^6) were inoculated into a 35 mm culture dish and the cells were grown to high density overnight. After having been washed twice with PBS, the cells were incubated at 37°C for 1 hour in DMEM together with cholesterol sulfate-MβCD inclusion complexes. The cells were washed three times with ice-cold PBS (phosphate-buffered saline solution)/BSA (bovine serum albumin) (PBS containing 2 mg of BSA/ml) in order to remove inclusion complexes and the cells

were cooled on ice for 5 minutes before they were subjected to crosslinking. Routinely, in a standard crosslinking protocol, the membrane-impermeable crosslinker bis(sulfosuccinimidyl)suberate (BS^3) is used, diluted down to 0.5 to 1 mM, in PBS. Cells were incubated with a BS^3 solution at 4°C for 45 minutes. Unreacted crosslinking agent was quenched, at 4°C for 15 min, with 1 mM glycine dissolved in PBS. The cells were subsequently lyzed with a Triton X-114-containing buffer and a detergent-rich phase was prepared in order to enrich GPI anchor proteins. The proteins were precipitated from the detergent-rich phase, fractionated on an SDS-PAGE gradient gel and blotted onto nitrocellulose, and GH-DAF was visualized using anti-GH antibodies, HRP-conjugated secondary antibodies and ECL.

The crosslinking of GH-DAF was significantly decreased, in a similar manner to that occurring in association with GM_1 treatment.

Example 2

Using gangliosides to alter the detergent solubility of GH-DAF

A frequently employed criterion for investigating the association of a protein with microdomains is its resistance to extraction by nonionic detergents, such as Triton X-100, at 4°C (Brown et al., Cell 68 (1992), 533-544). The detergent solubility of GH-DAF was therefore investigated after the cells had been loaded with gangliosides. It was found that the solubility of GH-DAF in TX-114 was significantly increased from 51 ± 4% to 83% ± 10% and 76% ± 8% in GM_1-loaded cells and in bbG-loaded cells, respectively (figure 4A).

These results demonstrated that gangliosides which are inserted into the plasma membrane displace GPI-APs from

lipid microdomains. Consequently, gangliosides which are bound to proteins which are protruding from the surface of the plasma membrane either do not exert an influence on, in particular inhibit, the crosslinking by means of steric hindrance, or only do this to a very slight extent. It is known that a decrease in the cholesterol content in cell membranes increases the solubility of GPI-APs in nonionic detergents (Cerneus et al., J. Biol. Chem. 268 (1993), 3150-3155; Hanada et al., J. Biol. Chem. 270 (1995), 6254-6260; Scheiffele et al., EMBO J. 16 (1997), 5501-5508). The solubility of GH-DAF in association with an extraction with TX-114 was shifted to 80% ± 7% after the membrane cholesterol had been extracted with CD (figure 4B). By contrast, stabilizing the microdomains by means of an antibody-induced crosslinking reduces the solubility of GH-DAF down to 34% ± 7% (figure 4B).

The results demonstrate that using exogenous gangliosides to modulate or alter the lipid content in the cell membrane modulates both the cluster behavior of GPI-APs and their ability to be solubilized in TX-114 at 4°C.

Example 3

Effect of gangliosides on microdomains

As was demonstrated above, loading MDCK-GH-DAF cells with gangliosides displaces GPI-APs from the rafts. In order to exclude the possibility that this effect arises partially as a result of detergent-like properties possessed by gangliosides, use was made of n-octyl-β-D-glucopyranoside (OG). This detergent resembles gangliosides chemically (it contains a carbohydrate head group, for example) and is known to solubilize GPI-APs completely when it is used above the critical micelle concentration (20-25 mM). MDCK GH-DAF cells were incubated with varying concentrations of OG

and crosslinked with BS^3. No influence on the crosslinking of GH-DAF was observed when OG was used at the same concentrations as the gangliosides (10-100 µM). Only a minimal inhibition of the crosslinking was observed even when cells were incubated with an 100-fold higher concentration of OG (10 mM) (figure 5; the inhibition amounted to 3.5% ± 0.7%). Remarkably, the membranes remained intact even under these circumstances since caveolin-1, which is a protein membrane whose N terminus and C terminus are both arranged towards the cytoplasm, was not crosslinked. It was only when OG was used above its critical micelle concentration (20 mM) that caveolin-1 was found as a crosslinked product of high molecular weight. The treatment of the MDCK GH-DAF cells with 100 µM GM_1 did not lead to caveolin-1 becoming crosslinked. These data show that the inhibition, by gangliosides, of the crosslinking of GH-DAF does not occur due to the membrane being solubilized as a result of any detergent-like properties possessed by gangliosides.

Example 4

Using gangliosides to inhibit the crosslinking of GH-DAF

This example demonstrates that inhibiting the clustering of GPI-anchored proteins is a specific property of gangliosides. A lipid which is not a constituent of the sphingolipid-cholesterol micro-domains should not have any effect on the crosslinking and solubility of GH-DAF. Ideally, phosphatidyl choline molecules, such as those which are found in cell membranes, for example, should be used for such a control experiment. However, these lipids cannot be introduced into the membrane by adding them exogenously. For this purpose, therefore, MDCK GH-DAF cells were incubated with a partially water-soluble,

fluorescent-labeled analog of phosphatidyl choline (NBD-C_6-HPC). NBD-C_6 lipids are known to reach the plasma membrane rapidly at low temperature, and their accumulation in the plasma membrane can be easily observed by means of immunofluorescence microscopy (Kean et al., J. Cell. Biol. 123 (1993), 1403-1419). The insolubility of NBD-C_6 HPC in detergents was then investigated. Cells were loaded with NBD-C_6-HPC and a detergent extraction was carried out at 4°C using Triton X-114. More than 99% of the NBD-C_6-HPC was isolated from the detergent-soluble fraction, thereby indicating that the lipid is not associated with the microdomains. No change in the crosslinking pattern was observed after incubating with various concentrations of NBD-C_6-HPC. The crosslinking efficiency was 73% ± 5% in cells treated with 100 µM NBD-C_6-HPC as compared with 73% ± 6% in control cells (figure 6A). NBD-C_6-HPC did not alter the solubility of GH-DAF (54% ± 2% soluble), either (figure 4A).

In order to determine whether the inhibition of the crosslinking of GH-DAF with gangliosides is reversible, MDCK GH-DAF cells were loaded with 100 µM bbG and subsequently incubated for 6 hours in a serum-containing medium prior to the crosslinking. As shown in figure 6B, 6 hours of incubation with a serum-containing medium were sufficient in order to restore the crosslinking pattern of GH-DAF almost completely. The crosslinking efficiency was 6% ± 1% less than in the control cells.

The insolubility of GH-DAF in the detergent TX-114 was likewise restored by incubating bbG-loaded cells with a serum-containing medium for 6 hours (61% ± 4% soluble) (figure 4A).

This example shows that the inhibition of the crosslinking of GH-DAF by gangliosides is specific and reversible.

Example 5

The distribution of GH-DAF is not altered by gangliosides

Sphingolipid-cholesterol microdomains or rafts are small and highly dynamic structures which cannot be resolved by conventional microscopic techniques. An examination was carried out to determine whether loading the cells with gangliosides leads to a global rearrangement of the plasma membrane and thus of the GPI-APs. The distribution of GH-DAF on MDCK cells, with or without loading with bbG, was analyzed by means of immunofluorescence labeling. In order to prevent a redistribution of GH-DAF after the fixing had taken place, a formaldehyde fixing was used, with this fixing being combined with a subsequent fixing with methanol (Harder et al., J. Cell. Biol. 141 (1998), 929-942). Incubating the cells with 100 µM bbG for 1 hour prior to the fixing, and labeling with antibody, had no effect on the diffuse distribution of GH-DAF on the cell surface (figures 7A and B). The intracellular staining stems from the arrangement of the protein in the Golgi apparatus and/or from the endosomal arrangement. A decrease in the membrane content of cholesterol, effected using 10 mM CD, did not lead to any detectable changes in the distribution of GH-DAF, either (figure 7C). By comparison, the addition of anti-GH-DAF antibodies before the fixing took place led to a speckled distribution of the immunofluorescence signal (figure 7D). These results demonstrate that the inhibition of the crosslinking by gangliosides does not occur because of any rearrangement of the membrane.

Example 6

Loading cells with gangliosides alters the autophosphorylation state of protein kinases

This example demonstrates that there is a correlation between the administration of gangliosides to MDCK GH-DAF cells and cell signal transmission processes. As an indication of signal transmission, the autophosphorylation state of cell protein kinases was measured, using an in-gel assay, after cells had been loaded with gangliosides. The conditions for the ganglioside loading were the same as those for the investigations on crosslinking. As can be seen from figure 8, the intensity of various bands increased significantly after adding gangliosides (arrow) whereas other bands only became visible following treatment with a ganglioside (arrow heads). In this connection, it is to be noted that chemical crosslinking with BS^3 on its own does not lead to any significant changes in the autophosphorylation state. These experiments demonstrate that, in addition to modulating the association of GPI-anchored proteins with microdomains, gangliosides are able to induce signal transmission processes in MDCK cells.

Example 7

Using gangliosides to inhibit the copatching of influenza HA and GH-DAF

Sphingolipid-cholesterol microdomains are small and highly dynamic structures which cannot be resolved by conventional microscopic techniques. An investigation was carried out to determine whether loading the cells with gangliosides leads to a redistribution of GPI-APs. The distribution of GH-DAF on MDCK cells, with or without loading with bbG, was analyzed by means of immunofluorescence labeling. In order to prevent a redistribution of GH-DAF after the fixing had taken

place, a formaldehyde fixing was used, with this fixing being combined with a subsequent fixing with methanol (Harder et al., J. Cell. Biol. 141 (1998), 929-942). Incubating the cells with 100 µM bbG for 1 hour prior to the fixing, and labeling with antibody, had no effect on the diffuse distribution of GH-DAF on the cell surface (figure 8, panels A and B). The intracellular staining stems from the arrangement of the protein in the Golgi apparatus and/or from the endosomal arrangement. A decrease in the membrane content of cholesterol, effected using 10 mM CD, did not lead to any detectable changes in the distribution of GH-DAF, either (figure 7C). By comparison, the addition of anti-GH-DAF antibodies before the fixing took place led to a patch-like distribution of the immunofluorescence signal (figure 7D).

Incubating living cells simultaneously with antibodies directed against microdomain-associated proteins leads to the independently crosslinked components being redistributed into overlapping patches. However, copatching is only observed in the case of raft marker pairs and not in the case of pairs consisting of a raft marker and a non-raft marker; it can be concluded from this that attractive forces are present in the shared lipid environment of raft proteins.

An investigation was now carried out to determine whether the exogenous administration of gangliosides disturbs the physical linkage of GH-DAF to lipid microdomains. The trimeric transmembrane protein influenza hemagglutinin (HA) was expressed, as a protein marker associated with the microdomains, in MDCK GH-DAF cells.

The cells were incubated simultaneously, at 4°C, with a monoclonal anti-HA mAb and a polyclonal anti-GH Ab, followed by an incubation with the respective secondary

antibodies. A microscope analysis showed that HA and GH-DAF were copatching in the majority of the cells (60% copatching, 35% partial overlapping and 5% random distribution) (figures 9A-F). When the cells were incubated for 1 hour with 100 µM GM_1 prior to the antibody-induced crosslinking, only a partial coclustering of HA and GH-DAF was then observed in most of the cells (31% coclustering, 50% partial overlapping and 19% random distribution) (figures 9G-L). These results demonstrate that the attractive forces between patches of HA and GH-DAF are disturbed by administering gangliosides, showing that GPI-APs are displaced out of the lipid microdomains.

Example 8

Experimental procedures

The following procedures were used in the above examples:

Inserting lipids, reducing the content of cholesterol and treating cells with octyl glucoside:

Stock solutions of bovine brain gangliosides (bbG) or GM_1 (1-10 mM) were prepared in PBS. The cells were loaded with 10-100 µM gangliosides in DMEM at 37°C for 1 hour (Masserini et al., Biochemistry 29 (990), 697-701; Saqr et al., J. Neurochem, 61 (1993), 395-411). In order to remove excess lipid, cells were washed thoroughly with PBS which contained 2 mg of defatted BSA/ml (PBS/BSA). An ethanolic 0.5% stock solution of 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (NBD-C_6-HPC) was injected into PBS, while vortexing vigorously, in order to give a 1 mM solution. The insertion of NBD-C_6-HPC into membranes was carried out, at 8°C for 1 hour, using 10-100 µM NBD-C_6-HPC. In order to free MDCK GH-DAF cells from the cholesterol,

they were incubated, at 37°C for 1 hour, with 10 mM methyl-β-cyclodextrin (CD). For the octyl glucoside treatment, MDCK GH-DAF cells were incubated, at 37°C for 1 hour, with various concentrations of octyl glucoside dissolved in DMEM.

Detergent extraction:

The cells were washed with cold PBS and extracted with 1 ml of TX-114 lysis buffer at 4°C for 30 minutes. The cells were scraped off and samples were centrifuged at 15 000 g for 30 minutes and at 4°C. The supernatant (soluble fraction) was removed and the pellet (insoluble fraction) was resuspended in 1 ml of lysis buffer. Soluble and insoluble fractions were precipitated with 10% TCA for 1 hour on ice and centrifuged at 15 000 g for 15 minutes and at 4°C. The pellets were washed with acetone (-20°C) and subjected to further processing by means of SDS-PAGE and Western blotting.

Crosslinking, electrophoresis and Western blotting:

The recently described crosslinking protocol was used (Friedrichson et al., Nature 394 (1998), 802-805). In brief, cells were washed twice with cold PBS. The crosslinking was carried out at 4°C for 45 minutes using 0.5 mM BS^3. Unreacted crosslinker was quenched, at 4°C for 15 minutes, with 50 mM glycine. The cells were lyzed, at 4°C for 20 minutes and at 37°C for 10 minutes, in a TX-114 lysis buffer (150 mM NaCl, 10 mM tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton-X-114 and protease inhibitors). The lyzates were cooled briefly on ice and clarified by being centrifuged for 15 minutes at 15 000 g. The supernatants were subjected, at 37°C for 5 minutes, to a temperature-induced phase separation. Aqueous and detergent-enriched phases were separated by centrifuging at 13 000 rpm for 3 minutes and at room temperature (RT). The aqueous phases were discarded and 0.9 ml of TX-114 washing buffer (150 mM

NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.06% Triton-X-114 and protease inhibitors) was added to the detergent phases. After a 15-minute centrifugation at 15 000 g and 4°C, the samples were subjected to a further phase separation. Finally, the detergent phases were precipitated with cold acetone (-20°C) and boiled in a Lämmli sample buffer at 95°C for 5 minutes. The proteins were separated using a 5-15% SDS-PAGE and transferred to nitrocellulose. Polyclonal antibodies directed against GH and the folate receptor, followed by the corresponding secondary antibodies and ECL, were used in order to detect GH-DAF and FR-GPI. Immunoreactive bands were quantified by scanning films densitometrically or using a LAS-1000 Luminescence Image Analyzer (Fujifilm, Germany). The data for each condition were averaged and the fluctuation expressed as a standard deviation. The experiments were carried out from 3 to 6 times. In the case of some gels, the corresponding X-ray films were scanned using Fotoshop software and the optical density of the immunoreactive bands was determined and printed out using MacBas (version 2.0).

Immunofluorescence:

Cells cultured on cover slips were washed 3 times with PBS and fixed with 3.7% paraformaldehyde in PBS at 8°C for 6 minutes and then with methanol at -20°C for 10 minutes. Subsequently, the cells were incubated with an anti-GH antibody in PBS at room temperature for 30 minutes, followed by an incubation with Cy3-conjugated anti-sheep-IgG at room temperature for 30 minutes. Three washing steps with PBS were carried out after each of the above incubations. The cover slips were embedded in Moviol and photographs were taken using a high-resolution C 4742-95 digital camera (Hamamatsu Photonics K.K., Japan), and the digital deconvolution was carried out using the digital

confocal Openlab routine (version 1.7.7) (Improvision, UK).

For the antibody-induced crosslinking experiments, the anti-GH antibody and the Cy3-conjugated anti-sheep-IgG were diluted in DMEM. The cells were incubated with the anti-GH antibody at 37°C for 20 minutes, washed with DMEM and incubated with Cy3-conjugated anti-sheep-IgG at 37°C for 20 minutes. The cells were fixed and embedded in Moviol as described above.

In-gel protein kinase assay:

Cells cultured in 6-well dishes were loaded with gangliosides or crosslinked, as described above, with BS^3, cooled on ice, washed 3 times with ice-cold PBS and lysed, at 4°C for 30 minutes, in 400 µl of lysis buffer (20 mM tris-acetate, pH 7.0, 0.1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM β-glycero-phosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% TX-100, 1 mM benzamidine, 2 µg of leupeptin/ml, 0.1% β-mercaptoethanol, 0.27 M sucrose and 0.2 mM PMSF) and scraped off using a rubber policeman. The lysates were clarified at 15 000 g for 15 minutes and aliquots were boiled, at 95°C for 5 minutes, with 5 × sample buffer. The samples were fractionated on 5-15% gels which had been polymerized with 5 mg of BSA/ml. The in-gel protein kinase assay was carried out in accordance with the method described by van Dam (van Dam et al., EMBO J. 14 (1995), 1798-1811) apart from the fact that 65 µCi of γ-^{32}P-ATP in 3 ml of buffer K were used per gel.

Viral infection and antibody-induced patching:

For an infection with the HA influenza virus, the virus was diluted in an infection medium (MEM, 50 mM Hepes, pH 7.3, penicillin (100 U/ml)/streptomycin (100 µg/ml), 0.2% BSA), and virus adsorption was carried out for 1 hour. The infection was continued for a further

2.5 hours. 100 µM GM_1, diluted in DMEM, were added to some cells during the last hour. For an antibody-induced patching of HA and GH-DAF, the cells were incubated with a polyclonal anti-GH antibody and a monoclonal anti-HA PR8 (H17L10), diluted 1:3 000 and 1:50, respectively, in DMEM. The incubation was carried out at 4°C for 1 hour. After a brief washing with PBS/0.2% BSA, the cells were incubated, at 4°C for 1 hour, with the corresponding FITC-coupled and Cy-3 coupled secondary antibodies. The cells were fixed and embedded and photographs were taken as described above. For the quantification, 60 randomly selected cells from two different experiments were stored as digital images. The cells were subdivided into three different categories in accordance with the extent of the overlapping between the patches: (1) coclustering (more than 70% overlapping); (2) partial coclustering; (3) random distribution.

The invention consequently relates in particular to the use of gangliosides and other substances for modulating sphingolipid-cholesterol microdomains. It can be used, for example, for developing active compounds which exert an influence on membrane transport, signal transmission, cell adhesion and/or enzymic processes in mammalian cells. The modulation can also prevent the entry of bacteria, pathogens and viruses into the cells.